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Influence of Epithelium and Isoprenaline Incubation on Responsiveness of Guinea-Pig Trachea to Methacholine

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Key Words
Epithelium · Adrenergic receptors · Asthma · Airway responsiveness · Desensitization

Abstract
There are reports regarding harmful effect of long-term use of β_2-agonist drugs on asthma severity and airway responsiveness. In the present study, the responses of guinea pig trachea with intact and denuded epithelium (groups 1 and 2, n = 10) to methacholine as EC_{50} were measured in tissues nonincubated or incubated with 10 μmol/l isoprenaline during the resting period. The same protocol was performed in groups 3 and 4 (n = 5 for each group) with an additional 30 min rest time after isoprenaline incubation. The response of trachea with denuded epithelium (groups 2 and 4) to methacholine was significantly higher than that with intact epithelium both in incubated and nonincubated conditions (groups 1 and 3, $p < 0.05$ to $p < 0.001$). Incubation with isoprenaline caused a significant reduction in the tracheal response to methacholine in both the denuded groups ($p < 0.005$ and $p < 0.001$) and intact epithelium groups ($p < 0.005$ for both cases). The reduction in tracheal responsiveness to methacholine due to incubation in epithelium denuded trachea (groups 2 and 4) was nonsignificantly greater than that of intact epithelium tissues. There was no difference between groups 3 and 4 with those of groups 1 and 2 in both incubated and non incubated conditions. The maximum contractility response to methacholine was not different between tracheal chains with denuded and intact epithelium and did not change due to incubation with isoprenaline. The results of this study indicate reduction of tracheal response to methacholine due to incubation of tissues with isoprenaline, which was relatively more pronounced in epithelium denuded trachea.

Introduction

The most characteristic feature of asthma is airway hyperresponsiveness to a wide variety of inhaled physical, chemical, pharmacological and immunological stimuli, and the level of responsiveness has been shown to correlate loosely with the severity of asthma [1].

The effect of regular use of a β-agonist inhaler, one of the main drugs used by asthmatic patient on asthma severity and specially airway responsiveness (usually measured as response to methacholine) is a very important and controversial issue. Several studies showed increased airway responsiveness due to regular use of a β-agonist inhaler [2–5]. However, other studies showed no effect or

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even beneficial effect of this type of drugs on asthma severity and airway responsiveness [6–9]. Although asthmatic patients use β-agonist inhaler, the effect of this type of drugs was usually studied on isolated tissues by their incubation with β-agonists for a short period of time of one hour [10, 11].

Therefore, in the present in vitro study the effect of incubation of denuded and intact epithelium tracheal chain with isoprenaline on responsiveness to methacholine was studied to evaluate the effect of a β-agonist on responsiveness to methacholine.

The purpose of using denuded epithelium tracheal chain was to have a model of asthmatic airways because epithelial shedding of airway is a pathological feature of asthma [12]. Airway epithelial damage is known to be associated with airway hyperreactivity which is the most characteristic feature of asthma. In fact, several in vitro studies have shown that epithelial damage leads to increased bronchial responsiveness to different pharmacological agonists [13–15]. Serosal vs. mucosal application of agonist ligands also leads to increased bronchial responsiveness [16]; but denudation of epithelium abolished this increased responsiveness [17].

Methods

Tissue Preparations

Guinea pigs (500–700 g) were killed by a blow on the neck, and trachea were removed. Each trachea was cut into 10 rings (each containing 2–3 cartilaginous rings). All the rings were then cut open opposite the trachealis muscle, and sutured together to form a tracheal chain [13].

Tissue was then suspended in a 10-ml organ bath (organ bath 61300, BioScience Palmer-Washington, Sheerness, Kent, UK) containing Krebs-Henseleit solution of the following composition (mmol/l): NaCl 120, $NaHCO_3$ 25, $MgSO_4$ 0.5, KH_2PO_4 1.2, KCl 4.72, $CaCl_2$ 2.5 and dextrose 11. The Krebs solution was maintained at 37°C and gassed with 95% O_2 and 5% CO_2. Tissue was suspended under isotonic tension of 1 g and allowed to equilibrate for at least 1 h while it was washed with Krebs solution every 15 min.

Assessment of Tracheal Response to Methacholine

In each experiment a cumulative log concentration-response curves of methacholine (Sigma Chemicals, UK) induced contraction of tracheal chain was obtained by adding increasing concentrations of methacholine (0.1 μmol/l to 10 mmol/l) every 2 min. To obtain the curve, the percentage of contraction of the tracheal smooth muscle due to each concentration of methacholine in proportion to the maximum contraction obtained by the final concentration was calculated and plotted against log concentration of methacholine. The effective concentration of methacholine causing 50% of maximum response (EC_{50}) of methacholine in each experiment was measured and considered as tracheal response to methacholine.

The tracheal response to methacholine was studied on tracheal chains with four different groups of experiments as follows:

(1) Tracheal chains with intact epithelium (control group, n = 10).

(2) Tracheal chains with denuded epithelium. Epithelium of trachea was removed by inserting a moistened cotton wire into lumen of trachea and gently rubbing with a corkscrew motion 5–6 times (denuded epithelium group, n = 10).

All the pharmacological measurements in both groups were preformed in two different conditions in random order as follows:

(a) Nonincubated tissues.

(b) Incubated tissues with 10 μmol/l isoprenaline during a 1-hour resting period.

(3, 4) Groups 3 and 4 were the same as groups 1 and 2, but in incubated conditions. Pharmacological measurements were performed after an additional 30 min resting period without incubation (n = 5 for groups 3 and 4).

In all experiments responses were recorded on a kymograph (ET8 G-Boulitt, Paris) and measured after fixation. The study was approved by the ethical committee of our institutions.

Statistical Analysis

The data of tracheal response to methacholine (EC_{50}) and maximum contractility response to methacholine were quoted as mean ± SEM. The data of incubated and non-incubated tissues were compared using the paired t test. The data of tracheal chains with epithelium with those of denuded epithelium and the data of groups 3 and 4 with those of groups 1 and 2 were compared using unpaired t test. Significance was accepted at $p < 0.05$.

Results

Histology

A few pairs of tissues were selected at random for histological evaluation. Among the rubbed epithelium trachea, more than 90% of epithelium had been removed, while among the control trachea almost all of the epithelium was intact (fig. 1).

Tracheal Response to Methacholine

Cumulative log concentration-response curves of denuded tracheal chains in both incubated and non incubated conditions showed left ward shift compared to those of intact epithelium trachea (fig. 2). The mean value of EC_{50} in tracheal chains of the epithelium denuded trachea in groups 2 and 4 (3.93 ± 0.62 and 4.66 ± 0.49 μmol/l, respectively) was significantly lower than in the intact epithelium tissues in both groups 1 and 3 (9.36 ± 2.60 and 12.31 ± 1.28 μmol/l, respectively, $p < 0.05$ for group 2 vs. 1 and $p < 0.001$ for group 4 vs. 3) (table 1; fig. 3).

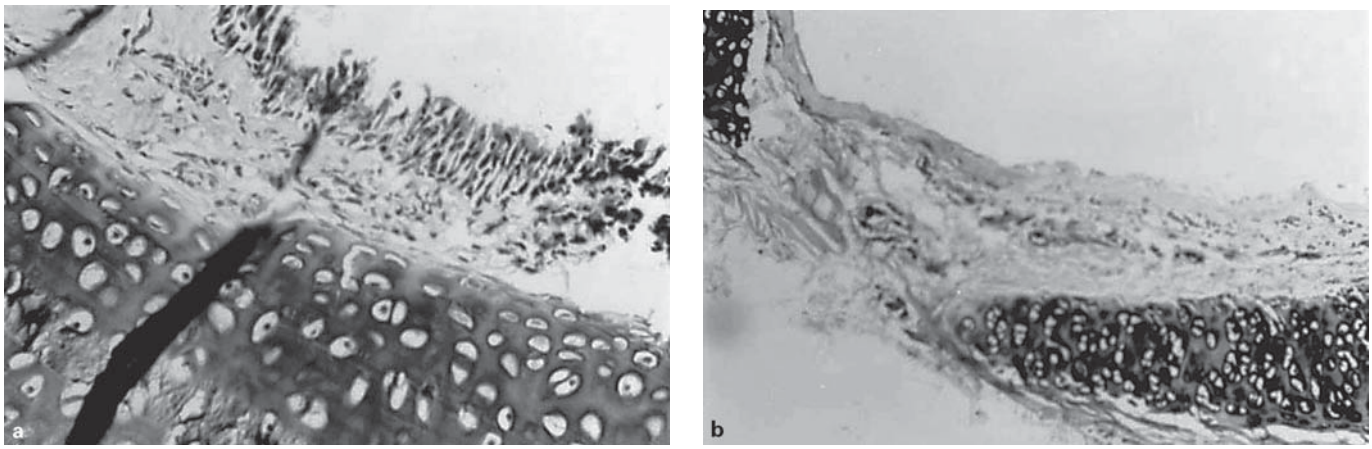

Fig. 1. Photographs of intact (**a**) and denuded epithelium thrachea (**b**).

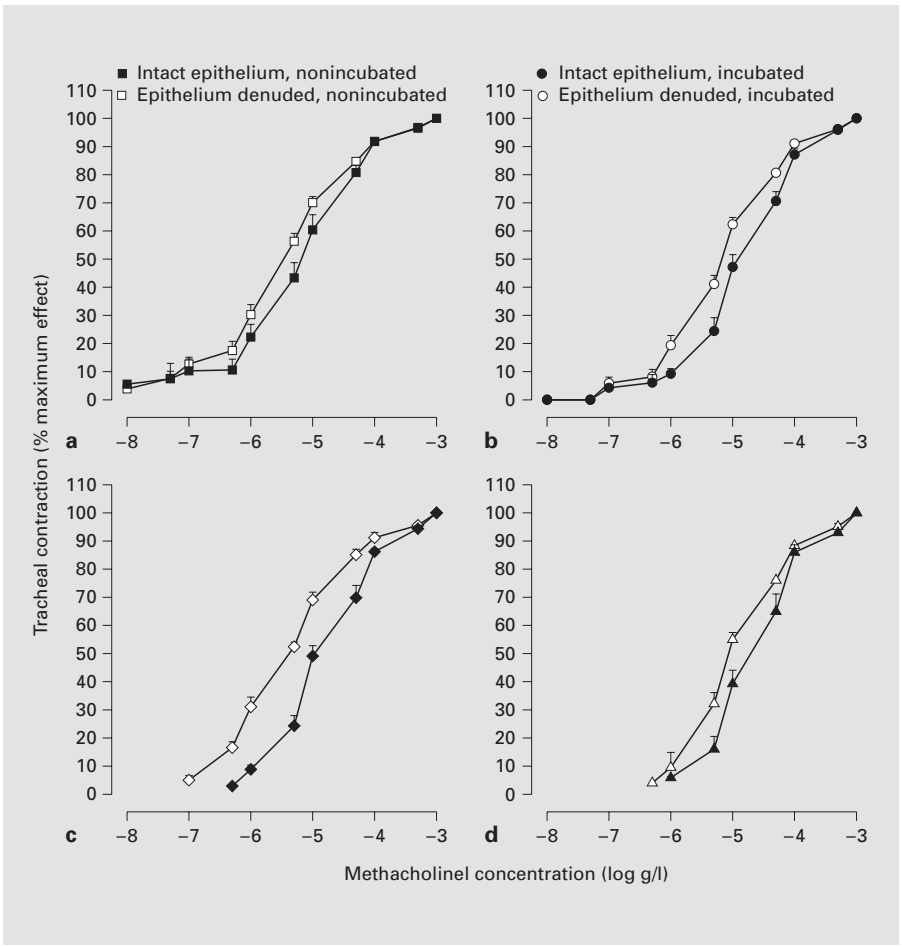

Fig. 2. Cumulative log concentration-response curves of methacholine-induced contraction in tracheal chains with intact (filled symbols) and removed epithelium (open symbols) in nonincubated (**a**) and incubated (**b**) samples in groups 1 and 2 (n = 10 for each group), and in groups 3 and 4 (**c**, **d**) (n = 5 for each group).

Table 1. Values of tracheal response to methacholine (EC_{50}, μmol/l) in four groups of experiments

	Groups 1 and 2	Groups 3 and 4	SD: groups 1 and 2 vs. 3 and 4
Intact epithelium			
Nonincubated	9.36 ± 2.60	12.31 ± 1.28	NS
Incubated	18.20 ± 4.03	19.66 ± 2.39	NS
SD: incubated vs. nonincubated	$p < 0.005$	$p < 0.005$	
Denuded epithelium			
Nonincubated	3.93 ± 0.62	4.66 ± 0.49	NS
Incubated	8.75 ± 1.33	8.67 ± 0.48	NS
SD: incubated vs. nonincubated	$p < 0.005$	$p < 0.001$	
SD: denuded vs. intact epithelium			
Nonincubated	$p < 0.05$	$p < 0.001$	
Incubated	$p < 0.05$	$p < 0.005$	

Values are presented as mean ± SEM. SD = Statistical differences; NS = nonsignificant difference. For groups 1 and 2, n = 10 and for groups 3 and 4, n = 5.

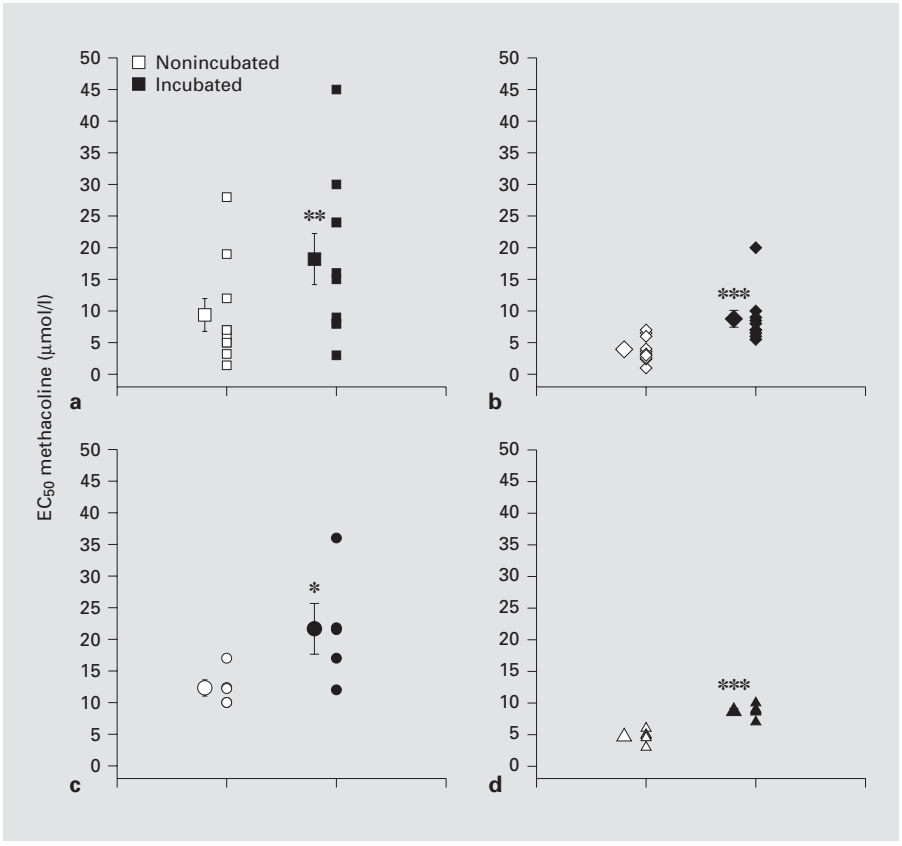

Fig. 3. Individual values and mean ± SEM (big symbols with bars) of tracheal response to methacholine (EC_{50}) in nonincubated (open symbols) and -incubated (filled symbols) samples tracheal chains with intact (**a**) and removed epithelium (**b**) in groups 1 and 2 (n = 10 for each group), and in groups 3 and 4 (**c**, **d**) (n = 5 for each group).

Table 2. Values of maximum contractility response (g) to methacoline in four groups of experiments

	Groups 1 and 2	Groups 3 and 4	SD: groups 1 and 2 vs. 3 and 4
Intact epithelium			
Nonincubated	0.21 ± 0.04	0.13 ± 0.04	NS
Incubated	0.18 ± 0.03	0.13 ± 0.02	NS
SD: incubated vs. nonincubated	NS	NS	
Denuded epithelium			
Nonincubated	0.18 ± 0.03	0.13 ± 0.03	NS
Incubated	0.17 ± 0.02	0.13 ± 0.03	NS
SD: incubated vs. nonincubated	NS	NS	
SD: denuded vs. intact epithelium			
Nonincubated	NS	NS	
Incubated	NS	NS	

Values are presented as mean ± SEM. SD = Statistical differences; NS = nonsignificant difference. For groups 1 and 2, n = 10 and for groups 3 and 4, n = 5.

Table 3. Changes in tracheal response to methacholine (EC_{50}) in four groups of experiments due to incubation of tissue to the β-agonist, isoprenaline

Groups	Intact epithelium	Denuded epithelium	SD: intact vs. denuded epithelium
1 and 2	130.34 ± 36.50	200.71 ± 59.50	NS
3 and 4	59.14 ± 10.68	90.58 ± 11.34	NS
SD: groups 1 and 2 vs. groups 3 and 4	NS	NS	

Values are presented as mean ± SEM of percent change relative to non incubated condition. SD = Statistical differences; NS = nonsignificant difference.

Change in Tracheal Responses to Methacholine due to Incubation of Tissues with Isoprenaline

Incubation of tissues lead to significant increase in EC_{50} both in the epithelium denuded preparations of groups 2 and 4 (8.75 ± 1.33 and 8.67 ± 0.48 μmol/l, respectively) and intact epithelium trachea in groups 1 and 3 (18.20 ± 4.03 and 19.66 ± 2.39 μmol/l, respectively, $p < 0.005$ to $p < 0.001$) (table 1; fig. 3). However, the EC_{50} of epithelium denuded trachea in groups 2 and 4 was also significantly lower than that of intact epithelium preparation of groups 1 and 3 in incubated conditions ($p < 0.05$ for group 2 vs. 1 and $p < 0.005$ for group 4 vs. 3), (table 1). The changes in EC_{50} due to incubation of tissues with isoprenaline in denuded epithelium trachea in groups 2 and 4 were nonsignificantly greater than those on intact epithelium tissues of groups 1 and 2 (table 3). There was no significant difference in EC_{50} between groups 1 and 2 with those of groups 3 and 4 in both incubated and non incubated conditions (table 1).

Maximum Contractility Response

There was no significant difference in maximum contractility response to methacholine between intact and denuded tracheal chains in both incubated and non incubated conditions. Incubation of both intact and denuded tracheal chains to isoprenaline did not affect maximum contractility response to methacholine. The maximum contractility responses of groups 3 and 4 were not significantly different with those of groups 1 and 2 (table 2; fig. 3).

Discussion

This study showed increased tracheal responsiveness to methacholine in epithelium denuded tracheal chains of guinea pig compared to intact epithelium trachea which is supported by the results of our previous study [18] and those of Holroyde [13] and Fedan et al. [16]. However, the maximum response to methacholine was not significantly different in epithelium denuded compared to intact epithelium tracheal chains. Histological evaluation confirmed the removal of epithelium in the epithelium denuded trachea.

The incubation of tissue with 10 μmol/l isoprenaline for one hour caused significant decrease in tracheal responsiveness to methacholine. However, the change in tracheal responsiveness to methacholine in epithelium denuded trachea was nonsignificantly greater than that of intact epithelium preparation. In fact tracheal responsiveness to methacholine in denuded tracheal chains was significantly greater than those of intact epithelium preparation in incubated conditions.

Epithelial damage is one of the pathological features of asthmatic airways [12, 19–21] which can lead to increased airway responsiveness, the main characteristic feature of asthma. Therefore, in the present study the effect of epithelium denudation and the phenomenon of desensitization by incubation of tissue with isoprenaline were examined on tracheal responsiveness to methacholine. The results showed that incubation of the tissues with isoprenaline lead to a relatively greater reduction in tracheal responsiveness to methacholine in the denuded epithelium tracheal chains.

In groups 1 and 2, postincubation measurement of tracheal responsiveness to methacholine was performed immediately after three consecutive washes of incubated tissue with Krebs solution. Therefore, the reduction in tracheal response to methacholine seems to be due to the presence of incubated isoprenaline at the receptor sites. Therefore, the effect of incubation of tissue with isoprenaline in tracheal response to methacholine was re-examined in groups 3 and 4. In these two groups, tissues were incubated with isoprenaline for 1 h following a 30-min rest at which tissues were washed with Krebs solution every 15 min. The post incubation measurements of tracheal response to methacholine were performed after the 30-min rest period. Therefore, with this design, the tracheal response to methacholine would not be affected or at least, will be less affected by the presence of incubated isoprenaline at the receptor sites. However, the reduction of tracheal response to methacholine in incubated tissues was very similar to those of groups 1 and 2. Therefore, the reduction in tracheal responsiveness to methacholine is not due to the presence of isoprenaline at receptor sites. In addition, the results of the present study showed that maximum contractile response to methacholine did not changed due to incubation of tissues with isoprenaline. This finding indicates that change in EC_{50} methacholine in incubated conditions is not due to functional antagonism.

While airway hyperresponsiveness is the main characteristic feature of asthma, the important question raised by the results of the present study is whether a decrease in trachea response to methacholine due to incubation with a β-agonist is an indication of beneficial or harmful effect of long-term administration of β-agonist therapy in asthma?

The reduction in tracheal response to methacholine in incubated tissues may indicate that incubation of tissue with β-agonist as well as long-term administration of β-agonist in asthmatic patients may lead to decreased airway responsiveness in asthma. Although the model of in vitro experiment used in the present study has not all the characteristics of asthma, the sustained improvement in pulmonary function tests, improvement of asthma symptom and no significant effect on airway responsiveness due to regular and long-term use of β_2-agonists have been shown [8, 9]. The anti-inflammatory effect of a β-agonist has been already shown [22], which could be the cause of the reduction of airway responsiveness due to incubation of tissue with isoprenaline seen in this study or administration of β-agonist in other studies [8, 9]. However, some investigators showed the harmful effects of this type of drugs on asthma severity and airway responsiveness [2–5]. Therefore, the effect of β-agonist drugs on airway responsiveness and its mechanism(s) should be examined in future studies.

In conclusion, the results of the present study showed a reduction of tracheal response to methacholine due to isoprenaline incubation and there was no significant difference between denuded and intact epithelium tracheal chain in this regard. Therefore, these results are very promising and may support those in vivo studies indicating that inhaled β-agonist drugs have no adverse effect on airway responsiveness in asthma.

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